Supplementary Materials, Shah et al

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Supplementary Methods.

Human tissue specimens: Normal breast organoid RNA was prepared as reported (10). Briefly, tissues from reduction mammoplasty performed at Johns Hopkins Hospital were mechanically macerated, and then digested overnight with hyaluronic acid and collagenase. The terminal ductal units were placed into suspension, and then isolated by serial filtration. Samples were treated with TRIzol and RNA extracted.

Fresh frozen primary breast tumors were obtained from the Department of Surgical Pathology tumor bank; specimens were from patients 45-55 years of age with localized disease, with positive estrogen receptor expression by immunohistochemistry as performed during routine staging at diagnosis, for uniformity of samples.

Metastatic breast carcinoma samples were obtained from the Rapid Autopsy Program at Johns Hopkins Hospital. All specimens were fast frozen at autopsy and stored at -80^{o}. Twenty 20-micron-thick sections were obtained from tumor metastases to the liver (for use on the tiling array) or other body sites (for validation studies). These slices were macerated with the BioMasher sample preparation device (Cartagen), with 350 uL of lysis buffer from the Qiagen RNEasy Mini Extraction kit. RNA extraction was completed with the flow-through from the BioMasher, as per the manufacturer protocol. 10 micron-thick H&E-stained tissue sections were made from all tumor samples and were confirmed to have at least 70% non-necrotic neoplastic epithelial cellularity before RNA extraction. Tissue samples were accessed after obtaining the appropriate approval from the Johns Hopkins Medical Institutions IRB Board.

RT-qPCR validation of gene expression: RNA from primary tissue samples was extracted using hte RNEasy Mini Extraction kit (Qiagen). RNA from cell lines was extracted from cells grown to 90% confluency in 6-well plates, using TRIzol reagant (Life Technologies). RNA was reverse-transcribed using Superscript III, and 1 µL of yield was used in for qPCR. Taqman® Gene Expression Assays for HOXB13 (Hs00197189_m1) and GAPDH (Hs99999905_m1) were used as primers and gene-specific fluorescent probes for PCR, using RampTaq polymerase (Denville Scientific) and supplied buffer. Primers for all other qPCR are listed in Supplementary Table 1 below, using the Maxima SYBR Green/ROX Master Mix (Fermentas), per manufacturer protocol. qPCR was performed per manufacturer protocol, using the Applied Biosystem 7500 Real-Time PCR system for 40 cycles. A detection threshold of 0.01 was set for determination of Ct for each reaction. For each sample, qPCR was performed to measure the gene of experimental interest (e.g. HOXB13) and GAPDH expression; each sample was tested in triplicate. The $\Delta\Delta C_t$ method (GAPDH used for normalization) was used to determine the expression of the gene in each reaction separately, using average lowest expression in organoid tissue as baseline. Relative expression was calculated as $2^{(-\Delta\Delta C_t)}$, and the three expression values averaged to determine HOXB13 expression in each sample.. Expression was calculated using the $\Delta\Delta C_t$ method as described above, using detection threshold of 0.01.

Plasmid Constructs, Viral Packaging, and Cell infection: A plasmid containing the full length cDNA of human HOXB13 in the pDNR-LIB vector (catalog number MHS1011-62759), a set of short-hairpin RNA microRNA (shRNAmir) lentiviral constructs targeted against the HOXB13 mRNA (RHS4533), and a nonsilencing lentiviral shRNAmir (RHS4346) were purchased from Open Biosystems. The HOXB13-pDNR-LIB vector was digested with the restriction endonuclease Sfi1 and the HOXB13 cDNA ligated into the retroviral vector pLPCX (Clontech) with T4 DNA ligase. The plasmid was transformed into Turbocells competent E. coli (Genlantis) per manufacturer protocol, which were then selected on LB agar supplemented with ampicillin (100 µg/mL). The resultant plasmid and the shRNAmir plasmids were amplified in LB broth with

ampicillin and purified with the QuantumPrep Plasmid Midiprep kit (Biorad). Sequences were confirmed with the Applied Biosystems 3730xl DNA Analyzer. The plasmids were then co-transfected with pCL-Ampho packaging vector (pLPCX) or psPAX2 and pMD2.G packaging vectors (lentiviral plasmids) into HEK-293T cells with Lipofectamine 2000 (Invitrogen), and viral supernatant collected at 36 and 72 hours.

MCF7 and T47D cells were treated with DEAE-dextran containing media, then the HOXB13-pLPCX, or pLPCX-vector control, viral supernatant for 24 hours. Infected cells were replated and treated with media containing puromycin, 0.2 μg/mL, for 2 weeks. Colonies were selected and HOXB13 expression evaluated; the colonies were also pooled and average HOXB13 expression evaluated.

The BT474 cell line was treated with DEAE-dextran containing media, then viral supernatant from either the nonsilencing shRNAmir (scramble) or constructs against HOXB13 for 24 hours. The cell lines were replated and treated with media containing puromycin, 0.1 μg/mL for 2 weeks. The propagated colonies were then re-infected with either shScramble, or HOXB13-targeting shRNAmir clones TRCN0000020844 (shHOXB13-1) or TRCN0000020845 (shHOXB13-2), and infected cells reselected with puromycin 0.2 μg/mL. The resultant pools (BT474-scramble, BT474-HOXB13-shRNA#1, and BT474-HOXB13-shRNA#2) were then tested to confirm HOXB13 expression. All cell lines were maintained in media supplemented with puromycin 0.2 μg/mL. Experiments were performed in media without puromycin.

Western blot analysis: Western blots were performed as previously described (29) $2x10^6$ cells were plated into 10 cm plates in complete media. After 24 hours, media was changed to either complete media with phenol red supplemented with 10% heat-inactivated fetal bovine serum, or phenol red-free media supplemented with 5% charcoal-stripped FBS. The medium was supplemented with either 2μM 4-hydroxytamoxifen (4-OH TAM) or vehicle alone. After four hours, cells were rinsed with PBS and lysed with RIPA buffer. 60 μg of extracted lysate were vertically electrophoresed on 4-12% Bis-Tris NuPage Novex Gel in MOPS SDS running buffer

(Invitrogen), then transferred to Hybond C Extra membrane (GE Healthcare). Membranes were stained with Ponceau stain to confirm protein transfer, then blocked with 5% powdered milk in PBS with 0.2% Tween-20 (PBST) for one hour at 25^{o}C. Membranes were probed with primary antibody in 5% milk/PBST at 25^{o}C for 2 hours, rinsed with PBST x 3, then probed with secondary antibody (either anti-rabbit-HRP or anti-mouse-HRP (GE Healthcare)) at 1:2000 dilution in 5% milk/PBST for 45 minutes. Membranes were rinsed with PBST x 3, then treated with ECL (or, for HOXB13, ECL Plus) Detection Reagent (GE Healthcare) for 1 minute. Membranes were exposed to Hyblot CL autoradiography film to determine protein expression. A list of antibodies used can be found in Table 2 below.

Chromatin Immunoprecipitation (ChIP): ChIP was performed with the EZChip kit (Millipore) according to protocol. 2.5 x 10^{6} cells were seeded into 15 cm plates and grown to 90% confluency. Protein and DNA were crosslinked with 1% formaldehyde in water and collected. Cells were sonicated with a Branson 450 ultrasonicator (4 cycles of 30 seconds each, 30% Duty cycle, output 4) on ice. The resulting lysate was either stored as input DNA or immunoprecipated with anti-HOXB13 antibody (F-9, Santa Cruz), normal mouse IgG, or anti-RNA Polymerase II (Millipore) overnight at 4^{o}C after preclearing, then complexes collected with ChIP-grade Protein G Agarose. The DNA crosslinking was reversed and DNA purified by spin column. 2 µL of the eluted DNA was then used for PCR, using primers designed to noted regions of the IL6 or CXCL12 promoter, extending 2000 bp upstream of the transcription start site (listed in Table 1 below). PCR was performed using BlueTaq polymerase (Denville Scientific), using the provided buffer, per manufacturer protocol, under the following cycle conditions: 95^{o}C for 2 min x 1 cycle, then 95^{o}C for 30 sec, 58^{o}C for 30 sec, and 72^{o}C for 30 sec x 30 cycles, then 72^{o}C for 2 min. Half of each reaction was run on 1% agarose/LB gel. Amplification in the input and anti-HOXB13 fractions and lack of amplification in the normal IgG fraction were indicative of specific binding of the probed DNA region by the HOXB13 antibody.

Tumor xenograft studies: 6-8 week old female athymic nude mice were used, and study approved by Johns Hopkins Animal Use Committee. E_2 and TAM pellets were made as previously described (30), using mixtures of 1:3 17-β estradiol:cholesterol or 1:1 TAM:cholesterol, and implanted subcutaneously (SC).

Cells were grown to 90% confluence, trypsinized, resuspended in PBS, and mixed 1:1 with Matrigel (BD Biosciences). Mice were anesthetized and the right 4^{th} mammary fat pad (MFP) exposed. On day 0, the indicated cancer cells were injected into the 4th mammary fat pads (mfp) ($1.5x10^6$ cells) or SC ($3x10^6$ cells) suspended in 0.1 mL of 1:1 PBS/Matrigel (BD Biosciences) in separate sets of mice. 6 mice each were injected with each cell type without drug.

In a second experiment, mice were implanted with E_2 pellets. Three days later, the mice were injected with cancer cells (24 each group) as described. After 3 weeks, 6 mice each were treated with either 1) TAM implants SC; 2) Rapamycin, dissolved as previously described(31), injected intraperitoneally (IP) with a loading dose of 9 mg/kg on day 1, then 3 mg/kg every other day, 3) TAM implants and rapamycin, or 4) no additional treatment.

Mice were measured weekly for tumor growth. After 6 weeks, mice were euthanized and tumors were sectioned and fast-frozen, or Formalin-fixed and paraffin-embedded and H&E slides made. Tumor volume was estimated by the calculation V=(length x width x height x 0.5236)mm^3.

Supplementary References:

1. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature2010 Apr 15;464(7291):1071-6.
2. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer2002 Jun;2(6):442-54.
3. Robinson SP, Jordan VC. Antiestrogenic action of toremifene on hormone-dependent, -independent, and heterogeneous breast tumor growth in the athymic mouse. Cancer Res1989 Apr 1;49(7):1758-62.
4. Granville CA, Warfel N, Tsurutani J, Hollander MC, Robertson M, Fox SD, et al. Identification of a highly effective rapamycin schedule that markedly reduces the size,

multiplicity, and phenotypic progression of tobacco carcinogen-induced murine lung tumors. Clin Cancer Res2007 Apr 1;13(7):2281-9.

Supplementary Tables.

Table S1: Primers used for PCR

Gene/Promoter Region	Forward Primer (5'-3')	Reverse Primer (5'-3')
ESR1	ATGATCAACTGGGCGAAGA	GGTGGACCTGATCATGGA
IL6	AGTGAGGAACAAGCCAGAGC	CAGGGGTGGTTATTGCATCT
CXCL12	GCATTGACCCGAAGCTAAAG	CCCCACTTTTTCTTCTCTGC
ESR1 Promoter Site 1	TGCGATGCACCTAATGTGT	ATCCCAAACACCCAACAGG
ESR1 Promoter Site 2	AACCTGTGGAAGGCATGAAC	TGCCAGGACCTCAGTTCT
ESR1 Promoter Site 3	TCCGTCTTTCGCGTTTATTT	ACGGGAGCAAGTGCAGTC
IL6 Promoter Site 1	TGCGTCCGTAGTTTCCTTCT	CCCATTGCCACTGAGTCTCT
IL6 Promoter Site 2	AGGGAGAGCCAGAACACAGA	CAGCACTTTGCATGTCTTG
IL6 Promoter Site 3	CAAGACATGCCAAAGTGCTG	GCCTCAGACATCTCCAGTCC

Table S2. Antibodies used for Western Blot

Antibody target	Vendor	Catalog number	Dilution
ERα	Santa Cruz	sc-543	1:1000
HoxB13	Santa Cruz	sc-28333	1:500
β-actin	Sigma Aldrich	A3853	1:3000
Total Akt	Cell Signaling	4691	1:1000
Total p70S6K	Cell Signaling	2708	1:1000
p-Akt (Ser473)	Cell Signaling	4058	1:1000
p-4EBP1	Cell Signaling	2855	1:1000
Total 4EBP1	Cell Signaling	9452	1:1000
p-STAT3	Cell Signaling	9131	1:1000
Total STAT3	Cell Signaling	4904	1:1000
p-p70S6K (Thr389)	Cell Signaling	9234	1:1000

Table S3 Treatment comparisons of tumor volumes at selected time points.

-MCF7-LPCX cell line, SC xenografts.

*Tumor growth inhibition was analyzed using a mixed effects model by assuming an exchangeable covariance structure to account for correlation among measurements taken on the same animal. Tumor volume measured at week 3 was considered as a baseline and controlled for in the model. Tukey's procedure was used to adjust for multiple comparisons.
All pair-wise comparisons between treatments were presented in the following table at 4, 5, and 6 weeks, respectively. Unadjusted p values were also provided for your reference. Suggest reporting the adjusted p values.

Treatment X	Treatment Y	Time (week)	Mean Difference	P value	Upper 95% CI	Lower 95% CI	Adjusted P value*	Adjusted Lower 95% CI*	Adjusted Upper 95% CI*
Control	Tam	4	28.8242	0.1023	-6.2766	63.9249	0.3283	-15.7993	73.4477
Control	Rapa	4	10.1391	0.5779	-27.2048	47.4829	0.9419	-37.3243	57.6025
Control	Rapa + Tam	4	24.2713	0.1691	-11.1843	59.7269	0.4893	-20.8086	69.3512
Tam	Rapa	4	-18.6851	0.3095	-56.0290	18.6588	0.7256	-66.1485	28.7783
Tam	Rapa + Tam	4	-4.5529	0.7919	-40.0085	30.9027	0.9932	-49.6328	40.5270
Rapa	Rapa + Tam	4	14.1322	0.4341	-22.7443	51.0087	0.8552	-32.7605	61.0250
Control	Tam	5	59.9784	0.0017	25.8032	94.1536	0.0028	16.9444	103.01
Control	Rapa	5	53.9644	0.0060	17.5765	90.3523	0.0148	8.1440	99.7848
Control	Rapa + Tam	5	69.5693	0.0005	34.8907	104.25	0.0005	25.8257	113.31
Tam	Rapa	5	-6.0140	0.7324	-42.4019	30.3740	0.9855	-51.8344	39.8065
Tam	Rapa + Tam	5	9.5910	0.5688	-25.0876	44.2696	0.9376	-34.1527	53.3346
Rapa	Rapa + Tam	5	15.6049	0.3752	-20.4239	51.6338	0.8001	-29.8313	61.0412
Control	Tam	6	91.1326	<.0001	56.0318	126.23	<.0001	46.5091	135.76
Control	Rapa	6	97.7897	<.0001	60.4459	135.13	<.0001	50.3263	145.25
Control	Rapa + Tam	6	114.87	<.0001	78.7522	150.98	<.0001	68.7281	161.01
Tam	Rapa	6	6.6571	0.7143	-30.6867	44.0010	0.9824	-40.8063	54.1205
Tam	Rapa + Tam	6	23.7348	0.1869	-12.3804	59.8500	0.5284	-22.4045	69.8741
Rapa	Rapa + Tam	6	17.0777	0.3552	-20.4091	54.5644	0.7812	-30.7957	64.9510

Table S4: Treatment comparisons of tumor volumes at selected time points -MCF7-HOXB13 cell line, SC xenografts

Treatment X	Treatment Y	Time (week)	Mean Difference	P value	Upper 95% CI	Lower 95% CI	Adjusted P value*	Adjusted Lower 95% CI*	Adjusted Upper 95% CI*
Control	Tam	4	89.4520	0.1507	-34.459	213.36	0.4594	-71.6140	250.52
Control	Rapa	4	79.1192	0.1661	-34.496	192.74	0.4959	-69.4320	227.67
Control	Rapa + Tam	4	94.6614	0.0847	-13.6730	203.00	0.2968	-46.9841	236.31
Tam	Rapa	4	-10.3328	0.8620	-130.47	109.81	0.9981	-167.02	146.36
Tam	Rapa + Tam	4	5.2094	0.9274	-110.42	120.84	0.9997	-145.53	155.95
Rapa	Rapa + Tam	4	15.5422	0.7797	-96.432	127.52	0.9921	-130.96	162.04
Control	Tam	5	-6.6286	0.9022	-118.36	105.11	0.9993	-147.97	134.71
Control	Rapa	5	182.32	0.0011	83.8828	280.76	0.0016	57.8023	306.84
Control	Rapa + Tam	5	192.28	0.0004	98.4141	286.15	0.0004	73.5454	311.01
Tam	Rapa	5	188.95	0.0015	82.9197	294.98	0.0026	54.8278	323.08
Tam	Rapa + Tam	5	198.91	0.0007	96.5687	301.25	0.0009	69.4550	328.36
Rapa	Rapa + Tam	5	9.9571	0.8308	-86.544	106.46	0.9964	-112.11	132.03
Control	Tam	6	-102.71	0.1008	-226.62	21.2027	0.3372	-263.78	58.3567
Control	Rapa	6	285.53	<.0001	171.91	399.14	<.0001	136.98	434.08
Control	Rapa + Tam	6	289.90	<.0001	181.56	398.23	<.0001	148.25	431.54
Tam	Rapa	6	388.24	<.0001	268.10	508.38	<.0001	231.55	544.92
Tam	Rapa + Tam	6	392.61	<.0001	276.98	508.24	<.0001	241.86	543.35
Rapa	Rapa + Tam	6	4.3720	0.9372	-107.60	116.35	0.9998	-142.13	150.87

* Tukey's procedure was used to adjust for multiple comparisons.

Table S5: Treatment comparisons of tumor volumes at selected time points
MCF7-LPCX cell line, MFP xenografts

Treatment X	Treatment Y	Time (week)	Mean Difference	P value	Lower 95% CI	Upper 95% CI	Adjusted P value*	Adjusted Lower 95% CI*	Adjusted Upper 95% CI*
Control	Tam	4	24.8565	0.1181	-6.7472	56.4601	0.3789	-15.9922	65.7051
Control	Rapa	4	16.1658	0.3264	-17.0526	49.3842	0.7500	-26.7669	59.0985
Control	Rapa + Tam	4	12.4757	0.4276	-19.3311	44.2825	0.8515	-28.6525	53.6039
Tam	Rapa	4	-8.6906	0.5954	-41.9091	24.5278	0.9494	-51.6234	34.2421
Tam	Rapa + Tam	4	-12.3807	0.4311	-44.1876	19.4261	0.8543	-53.5089	28.7475
Rapa	Rapa + Tam	4	-3.6901	0.8213	-36.9211	29.5409	0.9958	-46.6616	39.2814
Control	Tam	5	54.8326	0.0010	25.6087	84.0564	0.0014	17.8982	91.7669
Control	Rapa	5	46.7484	0.0050	16.0197	77.4771	0.0124	7.9115	85.5854
Control	Rapa + Tam	5	49.8880	0.0024	20.0872	79.6889	0.0052	12.0599	87.7162
Tam	Rapa	5	-8.0841	0.5872	-38.8129	22.6446	0.9454	-46.9211	30.7528
Tam	Rapa + Tam	5	-4.9445	0.7323	-34.7453	24.8563	0.9855	-42.7727	32.8836
Rapa	Rapa + Tam	5	3.1396	0.8346	-27.9046	34.1838	0.9966	-36.2374	42.5167
Control	Tam	6	84.8087	<.0001	53.2050	116.41	<.0001	43.9600	125.66
Control	Rapa	6	77.3310	<.0001	44.1126	110.55	<.0001	34.3983	120.26
Control	Rapa + Tam	6	87.3004	<.0001	53.8679	120.73	<.0001	43.7864	130.81
Tam	Rapa	6	-7.4776	0.6475	-40.6960	25.7408	0.9668	-50.4104	35.4551
Tam	Rapa + Tam	6	2.4917	0.8801	-30.9408	35.9242	0.9987	-41.0222	46.0057
Rapa	Rapa + Tam	6	9.9694	0.5619	-24.7432	44.6819	0.9357	-35.1804	55.1191

* Tukey's procedure was used to adjust for multiple comparisons.

Table S6. Treatment comparisons of tumor volumes at selected time points -MCF7-HOXB13 cell line, MFP xenografts

Treatment X	Treatment Y	Time (week)	Mean Difference	P value	Lower 95% CI	Upper 95% CI	Adjusted P value*	Adjusted Lower 95% CI*	Adjusted Upper 95% CI*
Control	Tam	4	17.0439	0.4612	-29.4779	63.5658	0.8780	-43.6953	77.7832
Control	Rapa	4	89.4076	0.0016	36.7246	142.09	0.0058	20.8538	157.96
Control	Rapa + Tam	4	88.3532	0.0005	41.4508	135.26	0.0019	27.1393	149.57
Tam	Rapa	4	72.3637	0.0050	23.4858	121.24	0.0204	8.5534	136.17
Tam	Rapa + Tam	4	71.3092	0.0029	26.0907	116.53	0.0122	12.1960	130.42
Rapa	Rapa + Tam	4	-1.0545	0.9650	-49.5865	47.4775	1.0000	-64.4334	62.3245
Control	Tam	5	44.7947	0.0330	4.0410	85.5485	0.1096	-6.7568	96.3463
Control	Rapa	5	182.66	<.0001	135.46	229.86	<.0001	122.96	242.37
Control	Rapa + Tam	5	183.94	<.0001	142.75	225.14	<.0001	131.83	236.05
Tam	Rapa	5	137.87	<.0001	95.0240	180.71	<.0001	83.6729	192.06
Tam	Rapa + Tam	5	139.15	<.0001	99.9171	178.38	<.0001	89.5227	188.77
Rapa	Rapa + Tam	5	1.2821	0.9501	-41.1578	43.7220	0.9999	-52.4024	54.9666
Control	Tam	6	72.5455	0.0033	26.0237	119.07	0.0133	11.8063	133.28
Control	Rapa	6	275.91	<.0001	223.23	328.60	<.0001	207.36	344.47
Control	Rapa + Tam	6	279.53	<.0001	232.63	326.43	<.0001	218.32	340.75
Tam	Rapa	6	203.37	<.0001	154.49	252.25	<.0001	139.56	267.18
Tam	Rapa + Tam	6	206.99	<.0001	161.77	252.21	<.0001	147.87	266.10
Rapa	Rapa + Tam	6	3.6187	0.8803	-44.9133	52.1507	0.9987	-59.7603	66.9976

* Tukey's procedure was used to adjust for multiple comparisons.